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# UNIVERSITÀ DEGLI STUDI DI TORINO

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# Peptide-Derivatized Dendrimers Inhibit Human Cytomegalovirus Infection by Blocking Virus Binding to Cell Surface Heparan Sulfate

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## Abstract

Dendrimers are hyperbranched synthetic well-defined molecules with a number of potential applications, especially in relation to the need for new antiviral agents. One subclass of dendrimers are peptide derivatized-dendrimers which consist of a peptidyl branching core and covalently attached surface peptide functional units. Few studies have addressed the potential uses of peptide dendrimers as direct-acting antiviral agents. Here, we report on the ability of two peptide dendrimers, SB105 and SB105\_A10, to directly and almost completely inhibit human cytomegalovirus (HCMV) replication in both primary fibroblasts and endothelial cells; the agents were also found to inhibit murine CMV replication, whereas they were not able to inhibit the adenovirus or vesicular stomatitis virus. The peptide dendrimers prevented adsorption of the HCMV to cells at 4° C, whereas SB104, a dendrimer with a different amino acid sequence within the functional group and minimal anticytomegaloviral activity, was ineffective in blocking HCMV attachment. In effect, SB105\_A10 bound to human cells through an interaction with cell surface heparan sulfate and thereby blocked virion attachment to target cells. These results indicate that the SB105 and SB105\_A10 dendrimers could provide a useful starting point for the development of novel molecules to block HCMV infection.

**Keywords:** Human cytomegalovirus, antivirals, peptide-derivatized dendrimers, in vitro infection, inhibition of viral attachment, heparan sulfate, GCV-resistant strains.

## 1. Introduction

Human cytomegalovirus (HCMV) is an important and ubiquitous opportunistic viral pathogen which generally causes asymptomatic infections in healthy individuals (Landolfo et al., 2003; Mocarski et al., 2006; Britt, 2008). However, HCMV infections are a major cause of morbidity and mortality among immunocompromised patients, such as recipients of bone marrow and solid-organ transplants and AIDS patients (Britt, 2008) for whom prolonged antiviral therapies are often necessary. The current therapy for the treatment of HCMV infections relies upon the use of six drugs: ganciclovir (GCV), valganciclovir, foscarnet (FOS), cidofovir (CDV), fomivirsen (ISIS 2922) and maribavir (Michel and Mertens, 2006; De Clercq, 2008). Despite the clinical benefits, the current standard therapy for HCMV disease also carries disadvantages, including dose-related toxicities and the existence of drug-resistant strains. Thus, a great need exists for the development of new anticytomegaloviral agents with reduced toxicity and that can also face the clinical problems resulting from the emergence of drug-resistant isolates (Michel and Mertens, 2006). In response to this urgent need for new antiviral agents, dendrimer-based molecules have been recognised as having a large number of potential therapeutic applications (Rosa Borges and Schengrund, 2005;

Greco et al., 2007). Dendrimers are highly branched macromolecules synthesized from a variety of polyfunctional cores in order to present multiple functional groups on the surface layer. Their hyperbranched structures enable a given molecular motif to be exposed in a highly multivalent fashion thus offering an efficient means of presenting multiple ligands, or sites of contact, on a single molecule (Cloninger, 2002; McCarthy et al., 2005). While the dendrimer core may vary, the surface functional groups in the antiviral dendrimers are represented by carbohydrates, anions or peptides (Rosa Borges and Schengrund, 2005). The development of peptide-derivatized antiviral dendrimers does not appear to have received a comparable amount of attention to that of the carbohydrate- or polyanionic-derivatized dendrimers. The aim of this study was to identify and characterize the anticytomegaloviral activity of novel peptide derivatized-dendrimers. They were derived from the M6 prototype, a tetrabranch dendrimer synthesized on a lysine core which tethers four 10-mer peptide chains in lysine  $\alpha$  and  $\epsilon$  positions (Pini et al., 2005). M6 has been shown to have an antibacterial activity against members of the Enterobacteriaceae and against clinical isolates of multidrug-resistant *Pseudomonas aeruginosa* (Pini et al., 2005). Its mechanism of action is thought to rely upon its interference with bacterial lipopolysaccharides (which causes the perforation of cell membranes without destroying external cell morphology) and with bacterial DNA (Pini et al., 2007). M6 was further shown to be endowed with high resistance against blood proteases, low hemolytic activity and low cytotoxic effect upon eukaryotic cells, thus making it a promising candidate for the development of new antibacterial and/or antiviral drugs (Pini et al., 2007).

## **2. Materials and Methods**

### **2.1 Cells, culture conditions, and viruses.**

Low-passage human embryonic lung fibroblasts (HELFs) were grown as monolayers in Eagle's minimal essential medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin sulfate. The EGFP-expressing reporter cell lines UL54 2F7 and UL112/113 1B4 (Luganini et al., 2008b) were cultured in monolayer in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin sulfate. Human umbilical vein endothelial cells (HUVECs), obtained by trypsin treatment of umbilical cord veins, were grown in endothelial growth medium (EGM-2; Cambrex Bio Science, Walkersville, MD) supplemented with 2% FBS, human recombinant vascular endothelial growth factor, basic fibroblast growth factor, human epidermal growth factor, insulin growth factor 1, hydrocortisone, ascorbic acid, heparin, gentamicin, and amphotericin B (1  $\mu$ g/ml each). Experiments were performed with cells between passages two and six. NIH 3T3 cells were grown as monolayers in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% donor bovine serum (Gibco-BRL), 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin sulfate. The HCMV strain AD169, obtained from the ATCC (VR538), was propagated in HELFs infected at a multiplicity of infection (M.O.I.) of 0.01 PFU/cell, incubated in minimal essential medium supplemented with 1% heat-inactivated FBS and cultured until a marked cytopathic effect could be observed. Virus stocks were then prepared by sonicating the cells, followed by centrifugal clarification and titration using a standard plaque assay on HELFs. HCMV TB40 UL32-EGFP is a recombinant HCMV TB40 strain in which enhanced green fluorescence protein (EGFP) is fused to the C-terminal end of the UL32 gene (Laib Sampaio et al., 2005). It was propagated on HELFs, pelleted through a 20% sorbitol cushion, and titrated as described above for HCMV AD169. HCMV VR1814 and HCMV AL1 are derivatives arising from clinical isolates recovered from a cervical swab from a pregnant woman (Revello et al., 2001) and the bronchoalveolar lavage fluid of a lung transplant recipient respectively. These strains were propagated in HUVECs and titrated by the indirect immunoperoxidase staining procedure on HELFs using a monoclonal antibody (MAb) reactive to the HCMV IE1 and IE2 proteins (clone E13; Argene Biosoft) (Revello et al., 2001).

HCMV VR5438 and TR are GCV-resistant strains isolated from AIDS patients undergoing GCV therapy and that developed CMV retinitis: their effective IC<sub>50</sub> are 18  $\mu$ M for VR5438 and 33  $\mu$ M for TR. Nucleotide sequence analysis of their UL97 gene revealed amino acid alterations of codon 595 (Leu-Phe) for VR5438 and the lack of codons 591-594 for TR (Gerna et al., 1998; Baldanti et al., 2002; Smith et al., 1998). VR5438 and TR strains were propagated in HELFs and titrated by the indirect immunoperoxidase staining procedure on HELFs using a monoclonal antibody (MAb) reactive to the HCMV IE1 and IE2 proteins (clone E13; Argene Biosoft) (Revello et al., 2001). The murine cytomegalovirus (MCMV) strain Smith (ATCC VR194) was propagated in NIH 3T3 cells and titrated by standard plaque assay. The vesicular stomatitis virus (VSV) serotype 'Indiana' and a clinical isolate of the adenovirus were propagated and titrated by standard plaque assay on HELFs.

## **2.2 Dendrimers and antiviral substances.**

Peptide grade reagents, resins and solvents were used for SPPS (solid phase peptide synthesis) and were supplied by Novabiochem (Darmstadt, Germany). The HPLC grade solvents used in the analytical and semipreparative courses were supplied by Sigma-Aldrich (St. Luis, MO). Dendrimer synthesis was carried out manually employing Fmoc/tBu chemistry, using 2-(1Hbenzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate/N,N diisopropylethylamine activation and hydroxybenzotriazole /HBTU/diisopropylethylamine in a ratio of 1/1/2. All acidic peptide-based dendrimers were synthesized on a preloaded (Fmoc2-Lys)2-Lys- $\beta$ -Ala Wang resin. Fmoc protection of amines was removed employing 40% piperidine in NMP. The side chain protecting groups were: 2,4,6-trimethoxybenzyl (Tmob) for Gln, trityl for Gln and Asn, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Pbf) for Arg; tert-butyl ether for Ser; tertbutyloxycarbonyl (Boc) for Lys and Trp. In particular, the Tmob protective group was preferred when Gln was directly linked to the lysine core, while trityl protection was used when Gln was present in the N-terminal. All acidic peptides were cleaved from the resins and deprotected by treatment with trifluoroacetic acid, water and triisopropylsilane (in the ratio of 95:2.5:2.5). Reverse phase HPLC analyses were performed in gradient mode using 0.1 % v/v TFA in water as solvent A, and 0.1 % v/v TFA in acetonitrile as solvent B. Solutions were run at 1 ml/min and detected at 210 nm. Separation was achieved on a Jupiter Proteo analytical C12 column (4.6  $\times$  250 mm) supplied by Phenomenex (Torrance, CA) using a gradient of 5-95 % solvent B over 16 minutes. Peptide were purified on a Jupiter Proteo semipreparative C12 column (10  $\times$  250 mm) using a gradient of 20-60 % solvent B over 15 minutes. The identity and the purity of the final products were confirmed by MALDI-TOF mass spectrometry using sinapinic acid as the acidic matrix. MALDI-TOF mass spectra were acquired in the positive ion mode. Mass calibration was performed with standard peptides covering the range 500-6000 m/z. All purified peptides (HPLCUV purity >95%) were lyophilized and then used in all assays as trifluoroacetate salts. The molecular weights of SB104, SB105 and SB105\_A10 dendrimers are 4506, 5194 and 4682,9 respectively. Fomivirsen (also known as ISIS 2922) is a 21-base phosphorothioate oligodeoxynucleotide (5'-GCGTTTGCTCTTCTTCTTGCG-3') complementary to the IE2 mRNA (Azad et al., 1993) was synthesized by Metabion International, Germany. All compounds were dissolved in phosphate buffer saline (PBS).

## **2.3 Antiviral assays.**

For the reduction of viral yield assay, both untreated cells and those incubated with different concentrations of peptides for 1 h before infection were infected with HCMV, MCMV, adenovirus, VSV or GCV-resistant HCMV clinical isolates (VR5438 and TR) at an M.O.I. of 1 PFU/cell. Following virus adsorption (2 h at 37°C), cultures were maintained in medium containing the corresponding peptides and then incubated until control cultures displayed extensive cytopathology. The cells and supernatants from the antiviral assay were then harvested and disrupted by sonication. The extent of virus replication was subsequently assessed by titrating the infectivity of the supernatants of cell suspensions on either HELFs (for HCMV, adenovirus and VSV) or NIH 3T3 cells (for MCMV) as previously described (Luganini et al., 2008a). Plaques were microscopically

counted and the mean plaque counts for each drug concentration were expressed as a percentage of the mean plaque count of the control virus. The number of plaques was plotted as a function of drug concentration; the concentrations producing 50 and 90% reductions in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>) were determined. Each experimental infection was carried out in duplicate and cell suspensions were titrated in duplicate.

To determine cell viability, HELFs were exposed to increasing concentrations of peptides. After 6 days of incubation, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988). For quantitative EGFP expression analysis, UL54 2F7 and UL112 /113 1B4 cells were seeded in 24-well plates at a density of 7 x 10<sup>4</sup> cells. After 24 h, cells were treated with either 10 µg/ml of one of the various dendrimers (Fig. 2) or with ISIS 2922 (5 µM); 1 h later, cells were infected with HCMV AD169 (M.O.I. of 5 PFU/cell). At 72 h post-infection (p.i.), the cells were washed with PBS and lysed with NP buffer (50 mM Tris-HCl, pH 7-8, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl<sub>2</sub>, 0.15% NP-40) at 4 °C for 30 min. Cell lysates were then centrifuged and the supernatants analysed for EGFP content using a Victor3 1420 Multilabel Counter, Perkin Elmer fluorescent microplate reader, with excitation and emission filters set at 485 and 535 nm respectively. Each experimental infection was carried out in duplicate and lysates were processed in duplicate.

#### **2.4 Immunoblotting.**

Whole-cell protein extracts were prepared as previously described (Caposio et al., 2007a). Proteins were separated by 8% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Filters were blocked overnight in 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20 and immunostained with the mouse anti-HCMV IE1 and IE2 MAb (clone E13; Argene Biosoft) (diluted 1:200), mouse anti-HCMV UL44 MAb (clone 1202; Goodwin Institute, Plantation, FL) (diluted 1:1000), mouse anti-HCMV UL99 MAb (clone 1207; Goodwin Institute) (diluted 1:1000) or mouse anti-actin MAb (Chemicon International) (diluted 1:2000). Immunocomplexes were detected using sheep anti-mouse immunoglobulin Ab conjugated to horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence (Super Signal; Pierce).

#### **2.5 Quantitative viral nucleic acid analysis.**

Real-time quantitative reverse transcription-PCR (RT-PCR) was performed on an Mx 3000 P apparatus (Stratagene) using SYBR Green as a non-specific PCR product fluorescence label as previously described (Caposio et al., 2007b; Lukanini et al., 2008a). After HCMV infection and cell treatment, total cellular RNA was isolated using the Eurozol reagent (Euroclone Ltd., United Kingdom) and RNA samples (1 µg) were then retrotranscribed at 42°C for 60 min in PCR buffer (1.5 mM MgCl<sub>2</sub>) containing 5 µM random primers, 0.5 mM deoxynucleoside triphosphates, and 100 U Moloney murine leukemia virus reverse transcriptase (Ambion) in a final volume of 20 µl. Reverse-transcribed cDNAs (2 µl), or water as the control, were then amplified in duplicate using the Brilliant SYBR Green QPCR master mix (Stratagene) in a final volume of 25 µl. Primer sequences were as follows: IE1 (sense, 5'-CAA GTG ACC GAG GAT TGC AA-3'; antisense, 5'-CAC CAT GTC CAC TCG AAC CTT-3'); IE2 (sense, 5'-TGA CCG AGG ATT GCA ACG A-3'; antisense, 5'-CGG CAT GAT TGA CAG CCT G-3'). Following an initial denaturing step at 95°C for 2 min to activate 0.75 U Platinum Taq DNA polymerase (Invitrogen), the cDNAs were amplified for 30 cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min). For quantitative analysis, semi-logarithmic plots were constructed of delta fluorescence versus cycle number and a threshold was set for the changes in fluorescence at a point in the linear PCR amplification phase (*C<sub>t</sub>*). The *C<sub>t</sub>* values for each gene were normalized to those for β-actin with the  $\Delta C_t$  equation. The level of target RNA, normalized to the endogenous reference and relative to that of the mock infected and untreated cells, was calculated using the comparative *C<sub>t</sub>* method with the  $2^{-\Delta\Delta C_t}$  equation.

#### **2.6 Attachment assays.**

The effect of dendrimeric peptides on viral attachment was evaluated by adsorption assay, as described by Shogan et al. (2006). To achieve this, prechilled HELF cell monolayers were treated with various concentrations of peptides or heparin for 1 h at 4 °C and infected with precooled 200 PFU of HCMV AD169 in the presence of the compounds for 3 h at 4°. Cells were then washed with cold MEM three times to remove unattached virus and compounds, overlaid with a 1.2% methylcellulose solution and incubated for 6 days at 37° C. Plates were then fixed and coloured with crystal violet for the microscopic counting of plaques. One lane per plate was used as a control to confirm that incubation at 4° C only allowed viral attachment and not viral entry. In order to establish this, the cells to which the virus had been attached at 4°C were treated with cold acidic glycine (0.1 M glycine, 0.150 M NaCl, pH 3.0) for 2 min before being covered with 1.2% methylcellulose medium. The number of plaques produced by these control wells was set to 100%. For microscopy analysis of HCMV TB40 UL32-EGFP binding to HELFs, cells were grown to semi-confluency on glass coverslips in 24-well plates. Then, prechilled cell monolayers were treated with 5 µg/ml of SB105-A10 or heparin (10 µg/ml), and infected with pre-cooled HCMV TB40 UL32-EGFP at an M.O.I. of 20 for 2 h at 4°C. Cells infected with EGFP-expressing virus were then gently washed twice with ice-cold PBS, fixed in 4% formaldehyde for 10 min on ice and examined by fluorescence microscopy following counterstaining with Evans blue (Sigma). Images were recorded with an Olympus Fluoview-IX70 inverted confocal laser scanning microscope.

### **2.7 Virucidal assay.**

To investigate the effect of dendrimeric peptides on viral infectivity (Shogan et al., 2006), dendrimers (5 µg/ml) were added to aliquots of HCMV AD169 (104 PFU) and the virus-peptide samples were incubated at either 4 or 37°C for various lengths of time. After incubation, the samples were diluted with culture medium to reduce the amount of peptide to a concentration that was identified as being non-active in the antiviral assay and the residual HCMV infectivity was then titrated on HELFs. Plaques were microscopically counted and the mean plaque counts for each peptide concentration expressed as PFU/ml on a log10 scale.

**2.8 Heparitinase assay.** HELF cell monolayers were pretreated with various concentrations of heparinase I or heparitinase I (Sigma) in PBS containing 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 0.1% glucose, and 0.1% bovine serum albumin or with buffer alone for 1 h at 37°C (Silvestri and Sundqvist, 2001). Cells were then washed three times with medium prior the analysis of HCMV infectivity or the binding of fluorescein isothiocyanate (FITC)-conjugated-SB105\_A10 dendrimer. Detection of bound fluorescent SB105-A10-FITC was performed on fixed cells (4% formaldehyde for 10 min on ice) by fluorescence microscopy following counterstaining with Evans blue (Sigma). Images were recorded with an Olympus Fluoview-IX70 inverted confocal laser scanning microscope.

### **2.9 Data analysis.**

All data were generated from duplicate wells in at least three independent experiments. The effects of the dendrimeric peptides at different concentrations were expressed as PFU/ml on a log10 scale and the mean plaque count for each drug concentration was expressed as a percentage of the mean plaque count of the control virus. Concentrations producing 50 and 90% reductions in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>) were calculated by linear regression using the computer program GraphPad Prism version 4.0.

## **3. Results**

**3.1 SB105 and SB105\_A10 inhibit HCMV replication in cell culture.** The effects of a minilibrary of dendrimeric peptides derived from the M6 prototype (Pini et al., 2005) (Fig. 1) upon the in vitro replication of HCMV AD169 laboratory strain were assessed using a cell-based assay that relies upon the UL54 2F7-EGFP and UL112/113 1B4-EGFP reporter cell lines (Luganini et al., 2008b). These cells were generated from the permissive U373-MG cell line in order to express EGFP under the control of the HCMV UL54 and UL112/113 early promoters and have been

validated as being a reliable tool for assessing virus-inhibitory effects in both concentration- and time-dependent fashions (Luganini et al., 2008b). As shown in Fig. 2, several peptide derivatized dendrimers showed moderate inhibitory activity towards HCMV AD169 replication. However, two of them, SB105 and SB105\_A10 decreased EGFP expression by over 90 % and were thus chosen for further analysis. Furthermore, an unbranched peptide with the same sequence as SB105 (SB 105 LIN, Fig. 2) showed a minimal inhibitory activity, thus suggesting that the dendrimer structure is required to confer an anticytomegaloviral activity to the specific amino acid sequence of the SB105 surface groups. Pretreatment of HELF cells with SB105 and SB105\_A10 peptides, 1 h before infection, produced a significant concentration-dependent inhibition of HCMV AD169 replication at 6 days p.i. (Fig. 3A). The IC<sub>50</sub> and IC<sub>90</sub> concentrations for HCMV replication were 0.22  $\mu$ M and 0.77  $\mu$ M for SB105 and 0.29  $\mu$ M and 1.04  $\mu$ M for SB105\_A10 respectively. The inhibitory effects of SB105 and SB105\_A10 were neither virus strain specific nor cell type specific since they were also observed in HELFs infected with the clinical isolate AL-1 (IC<sub>50</sub> 0.23  $\mu$ M for SB105 and 0.25  $\mu$ M for SB105\_A10) and in HUVECs infected with the endoteliotropic VR1814 strain (Fig. 3B) (IC<sub>50</sub> 0.24  $\mu$ M and 0.34  $\mu$ M respectively). Moreover, SB105 and SB105\_A10 did not significantly affect the viability of HELFs or HUVECs in the relevant range of concentrations as >90% of cells were still viable after 6 days of treatment with dendrimers at concentrations as high as 20  $\mu$ M (Fig. 3A and 3B); this demonstrates that the antiviral activity was not due to cytotoxicity of the target cells themselves. We then investigated whether the SB105 and SB105\_A10 dendrimers interfere with the replication of other viruses. As shown in Fig. 3C, the results obtained with MCMV were similar to those observed with HCMV. When preincubated with NIH3T3 cells, the dendrimers showed a significant inhibition of MCMV replication and the calculated IC<sub>50</sub>'s were 0.67  $\mu$ M and 0.98  $\mu$ M respectively. In contrast, the replication of a clinical isolate of adenovirus as well as of a VSV laboratory strain in HELF cells pre-treated with SB105 or SB105\_A10 was not significantly affected (Fig. 3C).

Altogether, these results demonstrate that SB105 and SB105\_A10 inhibit the in vitro replication of HCMV and MCMV, but not that of VSV or adenovirus, and that this antiviral activity is independent of cell type and virus strain.

### **3.2 SB105 and SB105\_A10 inhibit the replication of GCV-resistant strains of HCMV.**

In view of the occurrence of GCV-resistant strains during prolonged treatment therapies, the anticytomegaloviral activity of dendrimers in this context was evaluated by measuring the effect of SB105 and SB105\_A10 upon two GCV-resistant strains, TR and VR5438, that lack drug phosphorylation activity due to mutations in the UL97 gene (Smith et al., 1998; Gerna et al., 1998; Baldanti et al., 2002). Fig. 3D shows the effects of various concentrations of dendrimers upon viral titers determined by measuring the viral yield of virus-infected HELFs at 6 days p.i.. Replication in both GCV-resistant strains was significantly impaired by both SB105 and SB105\_A10, and their respective IC<sub>50</sub> values were 0.85  $\mu$ M and 0.49  $\mu$ g/ml for treatment of the TR strain, and 0.78  $\mu$ M and 1.2  $\mu$ M of the VR5438 strain.

### **3.3 SB105 inhibits the first phases of the HCMV replicative cycle.**

To obtain more insight into the mechanism of peptide derivatized-dendrimers antiviral activity, we investigated the effects of SB105 upon overall HCMV gene expression. In order to do this, total cell extracts were prepared from HCMV AD169-infected HELFs treated with SB105 for various lengths of time post-infection. The extracts were then analyzed for their contents of immediate-early (IEA), early (UL44) and late (UL99) proteins by immunoblotting with specific antibodies. Expression levels of IEA, UL44 and UL99 were assessed as a reflection of the levels of immediate early, early and late protein synthesis. SB105 inhibited the expression of all the assessed HCMV proteins at each of the time points analyzed (Fig. 4A). Then, RT-PCR was used to measure IE1 and IE2 mRNA levels in HCMV AD169-infected HELFs treated with SB105. As shown in Fig. 4B, and consistent with results above, the synthesis of both IE mRNAs was completely prevented by SB105 throughout the entire viral replicative cycle. This result supports the assertion that SB105 acts at a very early stage in the HCMV replicative cycle, that is, before the expression of IE genes.



Furthermore, incubation with SB105 or SB105\_A10 at 1  $\mu$ M after 2 h of virus adsorption did not significantly affect HCMV replication in HELFs, in contrast to the strong antiviral effect observed when the same dendrimers were added up to 3 h prior to infection (data not shown). This confirms that their antiviral activity targets the very early phases of the HCMV cycle, such as adsorption and/or entry.

### **3.4 SB105 and SB105\_A10 dendrimers impair HCMV attachment to target cells.**

To investigate whether the inhibitory activities of SB105 and SB105\_A10 are due to the inhibition of HCMV adsorption onto cells, prechilled HELFs monolayers were infected with HCMV AD169 in the presence of SB105, SB105\_A10, SB104 (a negative control of HCMV replication inhibition; Fig. 2) at various concentrations or heparin (a positive control for inhibition of viral attachment). After 3 h at 4°C, cells were washed to remove unattached virus and covered with a solution of 1.2% methylcellulose in order to measure the infectivity of HCMV that had successfully attached onto cells. As shown in Fig. 5, both SB105 and SB105\_A10 clearly impaired the attachment of HCMV in a concentration-dependent manner and to a similar degree as observed in the plaque reduction assay (Fig. 3A). In contrast, SB104 showed minimal inhibitory activity towards HCMV entry, consistent with the lack of any significant antiviral activity as seen in the plaque reduction assay (Fig. 2). As expected, heparin blocked the ability of HCMV to bind to HELF cells by inhibiting the interaction between the virus and cell surface HPSG (Compton et al., 1993; Isacson et al., 2008). To investigate further the mechanism of action of SB105 and SB105\_A10, we explored the possibility that they are able to interact with HCMV particles and thus inhibit viral infectivity. HCMV AD169 were incubated with 1  $\mu$ M of SB105, SB105\_A10 or SB104 at 4 or 37°C for various lengths of time. Following incubation, the virus/peptide mixtures were diluted with medium in order to reduce the concentration of dendrimers well below that able to inhibit HCMV replication; the residual HCMV infectivity was then titrated on HELFs (Shogan et al., 2006). As shown in Fig. 6, the pre-incubation of virions with dendrimers did not significantly affect HCMV AD169 at either 4 or 37°C and indicates that their inhibitory activities are not due to the irreversible inactivation of virions in solution. Thus, this result suggests that SB105 and SB105\_A10 are only able to inhibit HCMV infection when they present at the time when the virus attaches to the target cell.

### **3.5. SB105\_A10 dendrimer binds to cellular heparan sulfate.**

To further characterize the mechanism of action of dendrimers, we directly examined their binding to cell surface. As shown in Fig. 7A, a fine punctate cell surface pattern of green fluorescence was observed in HELFs incubated with a fluorescein isothiocyanate (FITC)-conjugated-SB105\_A10 (SB105\_A10-FITC) for 2 h at 4°C, thus indicating the successful binding to cells. To verify that the staining was specifically dependent on the dendrimer structure, binding of SB105\_A10-FITC was competed by the same dendrimer without FITC label, that clearly inhibited the interaction with cell surface (Fig. 7A). Moreover, the staining of HELFs from SB105\_A10-FITC was prevented by soluble heparin as well as by pretreatment with heparinase I or heparitinase I. Thus, these results indicate the ability of the dendrimer to bind cellular heparan sulfate (HS) (Fig. 7A). Furthermore, the attachment of HCMV UL32-EGFP, a recombinant reporter virus that expresses the EGFP protein fused to the capsid-associated tegument protein UL32 and thus generates green fluorescent virion particles (Laib Sampaio et al., 2005), was prevented in HELFs pretreated with both enzymes (Fig. 7B). Thus, the cleavage of cell surface HS by heparinase or heparitinase treatment was effective to block the initial interaction of HCMV with HSPGs (Compton et al., 1993; Isacson et al., 2008). Finally, as expected, SB105\_A10 treatment blocked the binding of HCMV UL32-EGFP virions to HELFs, further supporting the conclusion that dendrimers prevent HCMV attachment (Fig. 7B). Cumulatively, these findings indicate that the mode of action of SB105\_A10 towards HCMV infection involves the inhibition of virion binding to HPSGs on the surface of target cells.

## **4. Discussion**

Dendrimers are attractive in the terms of their potential use as therapeutic antivirals due to their small size (nanomolar), their ease of preparation and functionalization, and their ability to display multiple copies of surface groups (multivalency) required for biological recognition processes including the initial interactions which occur between an infecting virus and the target cell (Rosa Borges and Schengrund, 2005; Heegaard and Boas, 2006). To the best of our knowledge, this is the first study to demonstrate an anticytomegaloviral activity of peptide derivatized-dendrimers. The SB105 and its derivative SB105\_A10 showed a significant dose-dependent inhibitory effect upon infection of several HCMV strains (including GCV-resistant clinical isolates) by means of a mechanism in which the suppression of the initial attachment of virions to HSPGs on the cell surface seems to play a key role in their antiviral activities. Moreover, it appears that their antiviral activities are specific for Herpesviruses, since replication of both HCMV and MCMV as well as that of HSV-1 and HSV-2 (Luganini et al., unpublished results) was inhibited while negligible inhibition was observed for adenovirus and VSV. The antiviral activities of SB105 and SB105\_A10 are likely to stem from the specific amino acid sequence of the peptide surface groups attached to the dendrimer scaffold since variation of this sequence was found to reduce or abolish their inhibitory actions (Fig. 2). In fact, the screening of twelve distinct dendrimer compounds (Fig. 1) with differences in the amino acid sequences of their surface groups lead to the selection of SB105 and its derivative SB105\_A10 in which the Cterminal glutamine residue of the functional ASLRVRIKKQ group was removed. Herpesviruses are known to bind polyanionic molecules such as heparan sulfate polysaccharides which are usually linked to proteins to form the cell surface HSPGs. The initial and essential interaction of virions with HSPGs is a relatively conserved feature of herpesvirus entry (Isacson et al., 2008). Thus, numerous negatively-charged polyanions have been selected to bind herpesvirus envelope components and thereby block virion attachment and entry into target cells (Greco et al., 2007). Among these, dendrimers and dendrimer-like molecules have recently been generated with surfaces formed of negative charges and screened for potential anti-herpes activities. Indeed, *in vitro* analysis has revealed that sulfonated and carboxylated polylysine dendrimers effectively block the HSV-1 and -2 infection of cells and non-primate animal studies have shown that they were able to protect animals against an intravaginal HSV-2 challenge (Bourne et al., 2000; Bernstein et al., 2003; Gong et al., 2005). However, in addition to inhibiting the HSV attachment, the sulfonate dendrimers were also shown to act at a post-entry level by apparently inhibiting HSV DNA synthesis, which suggests that they could be used not only for prevention of HSV infection but also as inhibitors of viral replication (Gong et al., 2005). Sulfonated polylysine dendrimers, similar to those selected against HSV, were also found to inhibit HIV-1 infection and block vaginal SHIV (chimeric simian-human immunodeficiency-1 virus) transmission in pigtailed macaques (McCarthy et al., 2005; Rupp et al., 2007). On the basis of these results, SPL7013, a lysine-based dendrimer with naphthalene disulfonic acid surface groups, is currently being developed as a candidate topical microbicide, known as ViVagel, for the prevention of both HIV-1 and HSV-2 transmission (Rupp et al., 2007). However, the characteristics of the ASLRVRIKKQ peptide (which functionalizes the SB105 and SB105\_A10 dendrimers selected for their anticytomegaloviral activity) endow the molecule's surface with positive charges. It is therefore unlikely that they are able form complexes with HCMV envelope glycoproteins and therefore inhibit virion attachment in this mode. Consistent with this hypothesis, we observed that SB105\_A10 binds to human cells in a heparan sulfate-dependent manner since this interaction was prevented either by the homologous polysaccharide heparin or by heparinase/heparitinase treatment of target cells (Fig. 7). The interaction of SB105 and SB105\_A10 with HS polyanion thus lead to a block of infection as HS are the initial cellular attachment moiety for HCMV (Compton et al., 1993; Isaacson et al., 2008). HCMV is the principal pathogen in transplant recipients; it has also been implicated in vascular disorders, such as transplant vasculopathy, restenosis and atherosclerosis that are characterized by endothelial cell activation, inflammatory cell infiltration and smooth muscle cell proliferation; moreover, HCMV is recognized as the leading viral cause of birth defects (Britt, 2008). The standard therapy for HCMV disease is associated with considerable adverse side effects,

and prolonged treatment may lead to the emergence of drug-resistant strains. In addition, the antivirals currently used are not able to prevent the reactivation of latent HCMV infection or the expression of IE proteins which play crucial roles in viral pathogenesis and immunomodulation. The importance of IE functions and the inability of the antiviral therapies currently available to prevent their expression has led to the suggestion that the prevention of their expression and/or functions may provide an alternative strategy for the inhibition of HCMV reactivation, replication and immunopathogenesis (Michel and Mertens, 2006). The identification of novel anti-cytomegaloviral agents able to block either HCMV entry and/or gene expression at very early stages without causing major adverse effects is therefore needed. The potential use of compounds targeting virus attachment and entry could be hampered by the cell to- cell spread that characterizes recent clinical HCMV isolates (Mocarski et al., 2006). However, in the normal host the release of cell-free virus depends on the site of infection, and although cell-free virus transmission during hematogenous dissemination is thought to be unlikely because HCMV replication is highly cell-associated, cell-free virus is commonly found in body fluids such as urine, saliva and breast milk and often at high titers (Britt, 2008). Thus, molecules such as SB105 and SB105\_A10 that act by blocking virus attachment may represent an attractive target for antiviral drug development to prevent HCMV transmission via these excretions and to reduce disseminated infections in severely immunocompromised patients in which large amounts of cell-free infectious virus have been documented in the peripheral blood (Britt, 2008). Our results indicate that SB105 and SB105\_A10 may be attractive candidates for such a new class of antiviral drugs: drugs that exert their effects via a novel pathway targeting virus attachment and might have advantages in terms of lacking an induction of drug resistance. Their potent anticytomegaloviral activity in vitro warrants the execution of further studies to evaluate whether dendrimer treatment can also result in antiviral activity in vivo, through the use of animal models of acute infection. Since in vitro experiments have demonstrated that SB105 and SB105\_A10 inhibit MCMV replication (Fig. 3C), in vivo studies would be useful for validating their potential use in the prevention and/or control of HCMV infection.

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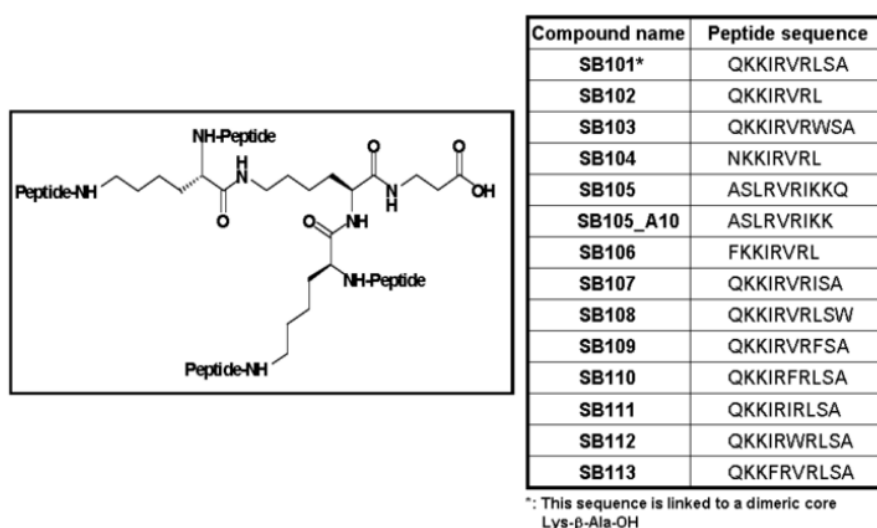
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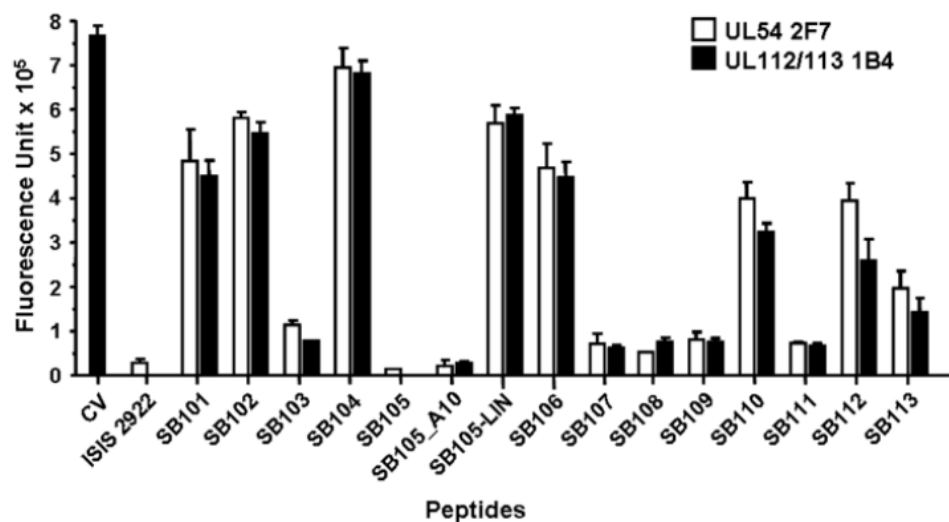
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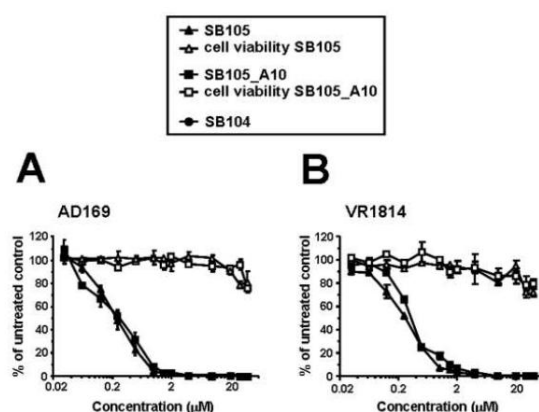
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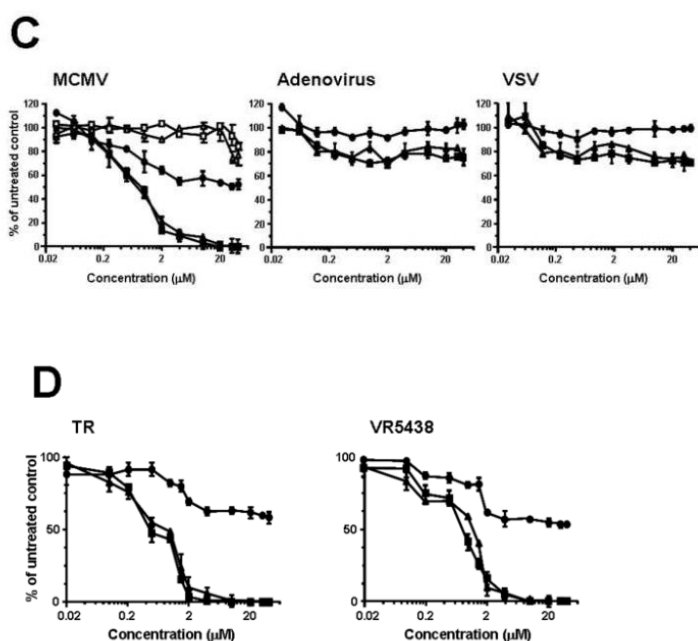


**Figure 1. Structures of the peptide derivatized-dendrimers. The generic structure of peptide is shown.** These molecules were synthesized by the addition of four 10-mer peptide chains to a tetrameric lysine central core. The amino acid sequence of each dendrimer peptide functional group is also shown.



**Figure 2. SB105 and SB105\_A10 dendrimers inhibit EGFP expression driven by HCMV early promoters in UL54 2F7 and UL112/113 1B4 reporter cell lines.** UL54 2F7 and UL112/113 1B4 cells were either mock-infected or infected with HCMV AD169 (M.O.I. of 5 PFU/cell) and, where indicated, the cells were pretreated and treated with 2  $\mu$ M of dendrimeric peptides, the SB105-LIN or with ISIS 2922 (5  $\mu$ M) 1 h prior to and during the infection period. At 72 h p.i., cell lysates were prepared and assayed for quantitative EGFP expression by automated fluorometry. The SB105-LIN is an unbranched synthetic peptide with the same sequence as the SB105 peptide surface group. The 21-bp antisense phosphorothioate oligonucleotide, complementary to the HCMV IE2 mRNA, fomivirsen (ISIS 2922), was used as the positive control of the inhibition of EGFP expression. The data shown in each column represents the mean  $\pm$  standard deviation (SD, error bars) of three independent experiments performed in duplicate.





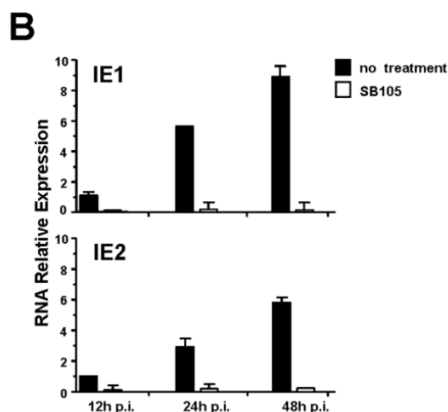
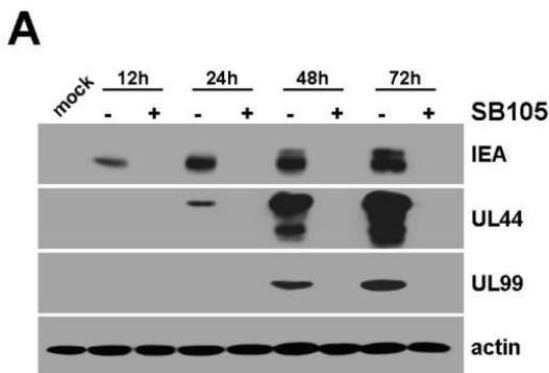
**Figure 3. Antiviral activity of SB105 and SB105\_A10 on productive HCMV replication. A) SB105 and SB105\_A10 inhibit HCMV AD169 replication.** HELFs were infected with HCMV AD169 (M.O.I. of 1 PFU/cell) and, where indicated, the cells were treated with increasing concentrations of dendrimers 1 h before virus adsorption and throughout the experiment until an extensive viral cytopathic effect was observed in the untreated controls. The extent of AD169 replication was then assessed by titrating the infectivity of supernatants of HELF suspensions using a standard plaque assay. Plaques were microscopically counted and the mean plaque counts for each drug concentration expressed as a percentage of the control mean count. The number of plaques is plotted as a function of drug concentration and the concentrations producing 50 and 90% reductions in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>) are shown. Data shown represent means  $\pm$  SD (error bars) of three independent experiments. To determine cell viability, HELF cells were exposed to increasing concentrations of peptides. After 6 days of incubation, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988).

**B) The replication of the clinical HCMV isolate VR1814 is blocked by SB105 and SB105\_A10 in endothelial cells.** HUVEC cells were infected with VR1814 (M.O.I. of 1 PFU/cell) and, where indicated, the cells were pretreated and treated with increasing concentrations of dendrimers 1 h prior to and during infection until an extensive viral cytopathic effect was observed in the untreated control. The extent of VR1814 replication was then assessed by titrating the infectivity of supernatants of cell suspensions on HELF cells by the IE antigen indirect immunoperoxidase staining technique. Plaques were microscopically counted, and the mean plaque counts for each drug concentration were expressed as a percent of the mean count of the control. The number of plaques was plotted as a function of drug concentration, and the concentrations producing 50 and 90% reduction in plaque formation (IC<sub>50</sub> and IC<sub>90</sub>) were determined. Data shown represent means  $\pm$  SD (error bars) of three independent experiments. HUVEC cells viability was determined as describe above.

**C) Effects of SB105 and SB105\_A10 on the replication of other viruses.** NIH3T3 cells were infected with MCMV Smith, while HELF cells were infected with either adenovirus or VSV at an M.O.I. of 1 PFU/cell. The cells were pretreated and treated with increasing concentrations of SB105 and SB105\_A10 1 h prior to and during viral infection until an extensive viral cytopathic effect was observed in the untreated controls. The extent of virus replication was then assessed by titrating the

infectivity of the supernatants of cell suspensions using a standard plaque assay. Plaques were microscopically counted and the mean plaque counts for each drug concentration are expressed as a percentage of the control mean count. Data shown represent the means  $\pm$  SD (error bars) of three independent experiments. NIH3T3 cells viability was determined as describe above.

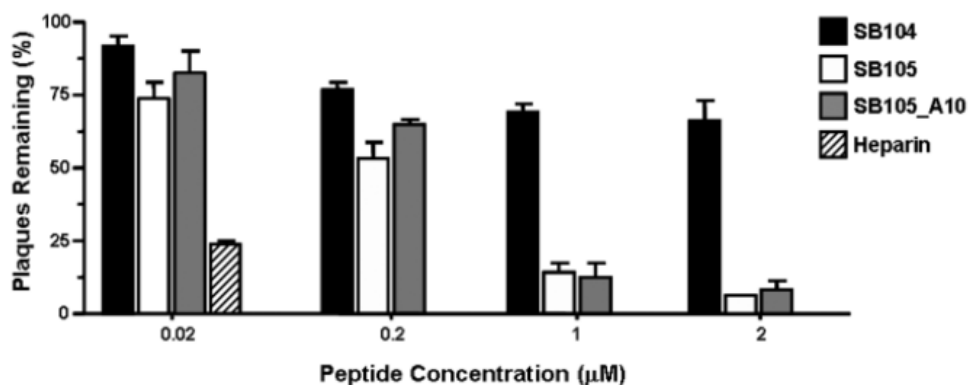
**D) Inhibitory effect of SB105 and SB105\_A10 on GCV-resistant HCMV TR and VR5438 strains.** HELF cells were infected with either HCMV VR5438 or HCMV TR (M.O.I. of 1 PFU/cell) or underwent mock infection. Where indicated, cells were treated with increasing concentrations of dendrimers 1 h before, during virus adsorption and throughout the experiment until an extensive viral cytopathic effect was observed in the untreated control. The extent of VR5438 or TR replication was then assessed by titrating the infectivity of supernatants of cell suspensions on HELF cells using the IE antigen indirect immunoperoxidase staining technique. The results shown represent the means  $\pm$  SD (error bars) of three independent experiments.



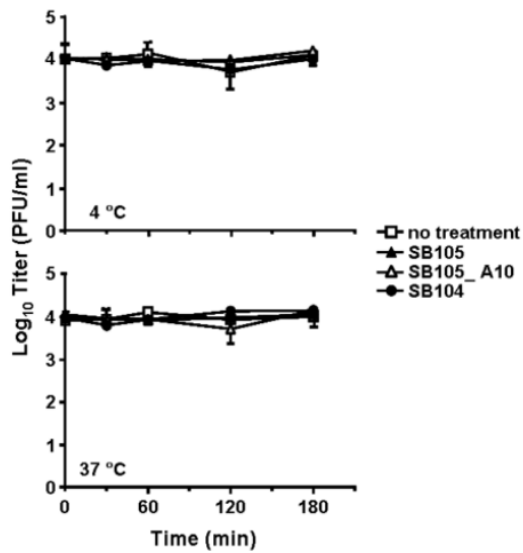
**Figure 4. SB105 prevents HCMV gene expression. (A) Effects of SB105 on HCMV IE, E and L proteins.** HELF cells were either infected with HCMV AD169 at an M.O.I. of 1 PFU/cell or mock infected; where indicated, the cells were pretreated and treated with 2  $\mu$ M SB105 1 h prior to and throughout the infection period. Total cell extracts were prepared at increasing times p.i., fractionated by SDS-PAGE (50  $\mu$ g protein/lane) and analyzed by immunoblotting with anti-IEA (IE1 and IE2), anti-UL44 or anti-UL99 MAb. Actin immunodetection with a MAb served as an



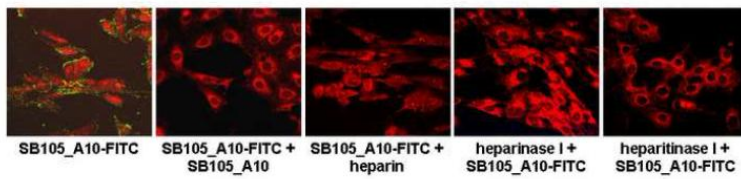
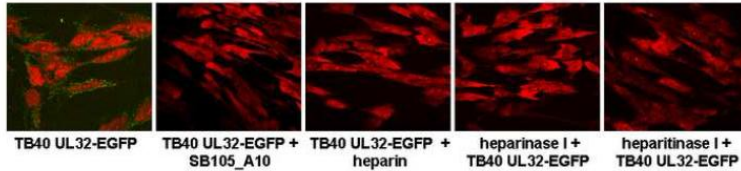
internal control. **(B) IE gene expression is inhibited in cells treated with SB105.** HELF cells were treated with SB105 and infected with HCMV AD169 as described in (A). Total RNA was isolated at the indicated time points after infection and subjected to reverse transcription. RT-PCR was then performed with the appropriate primers for IE1, IE2 and beta actin (as a control). For each timepoint, IE1 and IE2 RNA levels were normalized according to the expression of the actin gene. The data shown represent means  $\pm$  SD (error bars) of three independent experiments.



**Figure 5. SB105 and SB105\_A10 dendrimers prevent HCMV attachment.** Prechilled HELF cells were treated with various concentrations of SB105, SB105 A\_10, SB104 or heparin at 4 °C for 1 h and then infected with 200 PFU of HCMV AD169 for 3 h at 4°C in the presence of the compounds as indicated. After virus adsorption, cells were then washed with cold medium three times to remove unattached virus and compounds, covered with 1.2% methylcellulose containing medium and incubated at 37°C. At six days p.i., viral plaques were stained and counted. The data shown represent means  $\pm$  SD (error bars) of three independent experiments.



**Figure 6. Preincubation of SB105 and SB105\_A10 with virus does not affect HCMV infectivity.** HCMV AD169 aliquots (104 PFU) were incubated for various lengths of time in the absence of dendrimers, or in the presence of 1  $\mu$ M SB105, SB105 A\_10 or SB104 at either 4 or 37°C. Following various incubation times, the samples were diluted to reduce peptide concentrations to a level below that at which HCMV replication is inhibited and the virus was then titrated on HELF cells. Plaques were microscopically counted and the mean plaque count for each peptide concentration expressed as PFU/ml on a log<sub>10</sub> scale. The data shown represent means  $\pm$  SD (error bars) of three independent experiments.

**A****B**

**Figure 7. SB105\_A10 binds to cell surface heparan sulfate and inhibits HCMV adsorption. A) Effect of heparinase I or heparitinase I pretreatment of HELFs on binding of the labelled SB105\_A10-FITC.** HELFs monolayers were left untreated or treated with heparinase I (2.5 units/ml) or heparitinase I (1 units/ml) for 1 h at 37°C, washed with medium and chilled at 4°C. Then, SB105\_A10-FITC (1  $\mu$ M) was added alone, in the presence of a 10-fold molar excess of SB105\_A10 or heparin (10  $\mu$ g/ml) for further 1 h at 4°C. Cells were then fixed, counterstained with Evans blue, and examined by fluorescence microscopy. The experiment was repeated twice, and representative images are presented. **B) SB105\_A10 inhibits the attachment of the reporter strain HCMV TB40 UL32-EGFP.** HELFs were left untreated or digested with heparinase I (2.5 units/ml) or heparitinase I (1 units/ml) for 1 h at 37°C, washed with medium, chilled at 4°C and infected with HCMV TB40 UL32-EGFP (M.O.I. of 20) in the presence of SB105\_A10 (1  $\mu$ M) or heparin (10  $\mu$ g/ml) for 2 h at 4°C to allow for virus adsorption only. Cells were then fixed, counterstained with Evans blue, and examined by fluorescence microscopy to detect green fluorescent virus particles. The experiment was repeated twice, and representative images are presented.